

## Supplementary Material

### Molecular discrimination of structurally equivalent Lys63-linked and linear polyubiquitin chains

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## Supplementary Text

### Comparison with K63-linked diUb complexes

Two recent co-crystal structures of K63-linked diUb bound to an antibody fragment (Newton et al, 2008) and the AMSH-LP DUB (Sato et al, 2008) have revealed further features of K63-chain conformational flexibility. The structure of AMSH-LP, a JAMM (JAB1/MPN/Mov34 metalloprotease) family DUB specific for K63-linked chains, has revealed the molecular basis for its specificity (**Supp. Fig. 2A** and see main text). In contrast, the structure of a K63-specific antibody in complex with K63-diUb reveals that the diUb molecule is bent along its linker, with the antibody contacting the linkage residues (**Supp. Fig. 2B**). This conformation of K63-linked chains highlights their flexibility, although such antibody-based UBD does not occur *in vivo*.

### Comparison with K48-linked polyubiquitin

The structures of linear and K63-linked polyUb presented here are in marked contrast to the previously reported structures of K48-linked Ub dimers and Ub tetramers (Eddins et al, 2007) (**Fig. 2D**). K48 resides on an opposite surface of Ub relative to K63, and K48-linked Ub dimers adopt a compact structure with multiple hydrophobic contacts between their Ile44 patches. Longer polymers form from stacked Ub dimers (**Fig. 2D**). However, even the compact K48-linked chains display dynamic features (Trempe et al, 2005). Overall, K48-linked chains have a more compact polymeric structure compared to K63- and linear Ub chains (**Fig. 2D,E**).

## **Supplementary Material and Methods**

### ***Protein production and purification***

Ubiquitin (Ub) was expressed and purified according to (Pickart & Raasi, 2005), Ubc13 and Uev1a were expressed and purified according to (Zhang et al, 2005), E1 enzyme was expressed and purified from *Sf9* cells according to (Trempe et al, 2005). K63- and K48-linked Ub-chains were generated as described in (Komander et al, 2008), and the expression vector and purification protocol for linear tetraUb was described in (Reyes-Turcu et al, 2008). A20, AMSH and TRABID, as well as GST-tagged Ub binding domains of TRABID, NEMO, TAB2, cIAP1, ABIN2 and MUD1 were expressed in bacteria and purified using standard protocols or according to published procedures (Komander & Barford, 2008; McCullough et al, 2006; Tran et al, 2008; Trempe et al, 2005). CYLD was expressed in *Sf9* cells and purified according to (Komander et al, 2008). UCH-L2, UCH-L3, IsoT, USP2, and USP15 were obtained from BIOMOL (Exeter, UK).

### ***Data collection, phasing and refinement***

Diffraction data from diUb crystals was collected at the ESRF (Grenoble, France), beamline ID23. The crystals belong to the cubic space group  $P4_332$  with one diUb per asymmetric unit (**Supplementary Table I**). The structure was solved by molecular replacement using Phaser (McCoy et al, 2005) and monoUb (1ubq) as the search model. The initial phases of K63-linked diUb were interpreted with ARP/wARP (Perrakis et al, 1999) which built >95 % of the model. This model was subsequently refined with Refmac5 (Murshudov et al, 1997), incorporating TLS parameters, resulting in the final statistics in **Supplementary Table I**. The linear diUb crystals were solved by individual rigid body refinement of both Ub monomers, and subsequent rounds of simulated annealing in Phenix (Adams et al, 2002), while final refinement runs were performed in Refmac5.

### ***Mammalian expression and DUB assay***

HEK293 cells were seeded in 6-wells plates 18 hrs prior to transfection. One  $\mu\text{g}$  of either empty HA vector or HA-TRABID was transfected per well (2 wells per condition) using Lipofectamine 2000. Twenty-four hours following

transfection, proteins were extracted using NP-40 buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% NP-40, Complete EDTA-Free protease inhibitor tablets), and pre-blocked using 25  $\mu$ l of 50% protein G sepharose bead slurry (Zymed). 700  $\mu$ g of protein were immunoprecipitated using a Rat anti-HA antibody (Roche, 4  $\mu$ g/ml final concentration) for 2 hrs at 4°C. Immune complexes were captured with 50  $\mu$ l of 50% protein G sepharose bead slurry for 1 hr at 4°C. Beads were washed 3 times in NP-40 buffer, resuspended in 150  $\mu$ l of NP-40 buffer and incubated for 1 hr at 37°C with either 100 ng of K48, K63 or linear chains. 4X sample buffer was added to each reaction and samples loaded onto SDS PAGE. As an input control 50 ng of each linkage were loaded. Following transfer, the PVDF membrane was blocked in 5% milk (PBS, 0.1% Tween 20) and incubated with a rabbit anti-ubiquitin antibody (Millipore 07-375; 1/2000) and detected with a goat anti rabbit IgG-HRP antibody. 15  $\mu$ g of input protein was loaded onto a SDS PAGE gel and detected using a rat anti-HA (Roche: 1/2000) followed by goat anti-rat IgG-HRP antibody

### Supplemental references

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### Supplementary Table I

Data collection and refinement statistics. Values between brackets are for the highest resolution shell.

	K63-linked Ub-dimer	Linear Ub-dimer
<b><u>Data collection statistics</u></b>		
Beamline	ID23-2	ID23-1
Wavelength (Å)	0.8726	0.9537
Space Group	P4 <sub>3</sub> 32	P4 <sub>3</sub> 32
Unit Cell (Å)	<i>a,b,c</i> = 105.02, $\alpha,\beta,\gamma=90$	<i>a,b,c</i> = 105.57 $\alpha,\beta,\gamma=90$
Resolution (Å)	50-1.95 (2.06-1.95)	74.74-2.25 (2.37-2.25)
Observed reflections	86778	170734
Unique reflections	15023	9985
Redundancy	5.8 (5.3)	17.1 (5.8)
Completeness (%)	99.9 (100)	99.1 (96.9)
<i>R</i> <sub>merge</sub>	0.109 (0.591)	0.114 (0.537)
$\langle I/\sigma I \rangle$	11.4 (2.2)	27.4 (2.0)
Z	DiUb	DiUb
<b><u>Refinement statistics</u></b>		
Reflections in test set	756	478
<i>R</i> <sub>cryst</sub>	0.206	0.255
<i>R</i> <sub>free</sub>	0.250	0.277
Number of groups		
Protein residues	151	149
Ions	9	5
Water	77	26
Wilson <i>B</i> (Å <sup>2</sup> )	27.3	63.5
$\langle B \rangle$ protein (Å <sup>2</sup> )	42.8	43.5
$\langle B \rangle$ water (Å <sup>2</sup> )	38.9	46.7
RMSD from ideal geometry		
Bond length (Å)	0.015	0.014
Bond angles (°)	1.518	1.489
Main chain <i>B</i> (Å <sup>2</sup> )	0.878	0.670

## Supplementary Figure legends

### Supplementary Figure 1.

A) Crystal structure of K63-linked diUb in stereo representation. A  $2|Fo|-|Fc|$  electron density map contoured at  $1\ \sigma$  is shown for the linking residues. B) Crystal structure of linear diUb as in A. C) Superposition of K63-linked (orange) and linear (green) diUb as observed in mol A - mol B and mol B - mol C, respectively, aligned on the proximal molecule. Spheres represent the centre-of-mass for each ubiquitin moiety. While mol B and mol C are oriented slightly differently with respect to the proximal molecule through the flexible linker, importantly, the distance between individual Ubs is equivalent (39.5 Å vs 39.7 Å).

### Supplementary Figure 2.

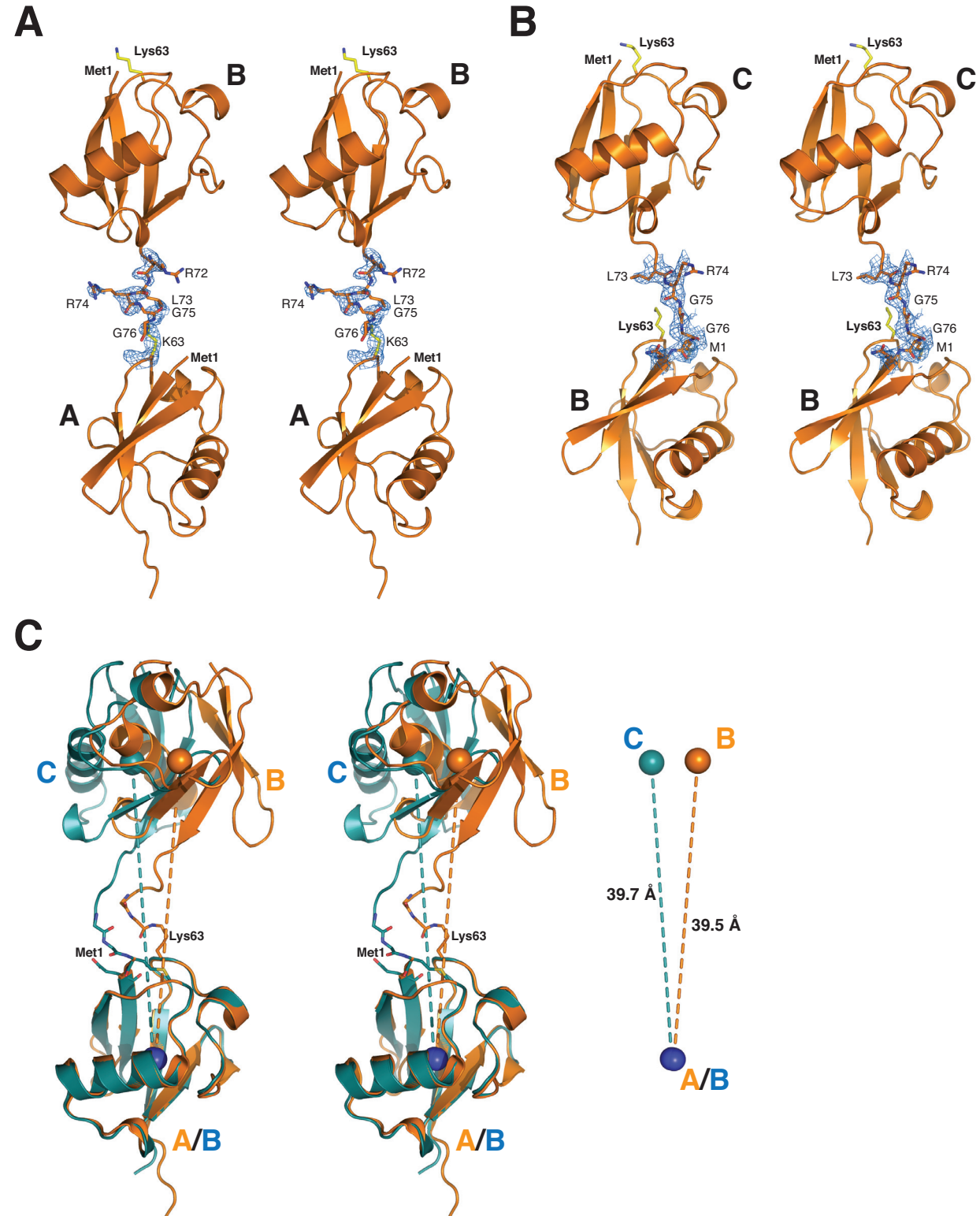
A semitransparent surface covers the Ub molecules in cartoon representation, and the position of the hydrophobic surface patch formed by Ile44-Val70-Leu8 is coloured in blue on the surface, indicated by arrows. The diUb molecules are aligned on the proximal Ub moiety. A) Structure of AMSH-LP bound to the extended K63 diUb (pdb id 2znv, (Sato et al, 2008). B) Structure of K63-diUb recognised by a K63-specific Fab fragment (pdb-id 3dvg, (Newton et al, 2008).

### Supplementary Figure 3.

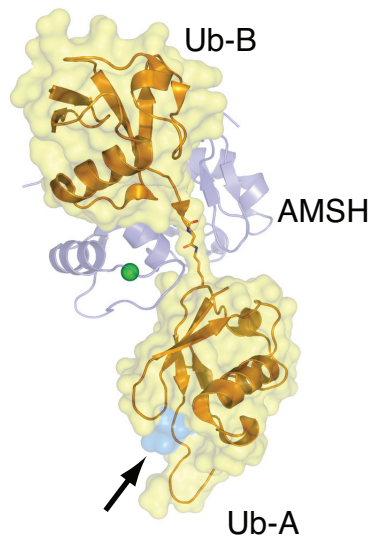
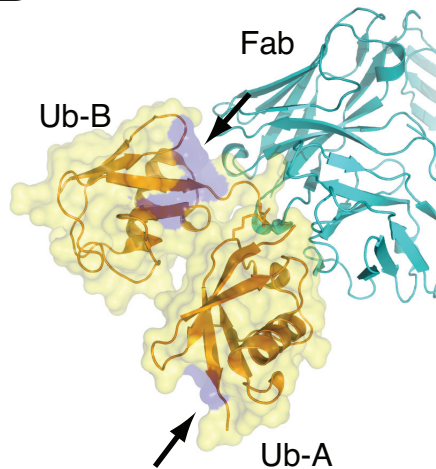
Membranes from the pull-down experiment stained in Ponceau Red after blotting. Analysed proteins are labelled by arrows. The small amount of tetraUb used in the experiment is undetectable by Ponceau Red.

### Supplementary Figure 4.

Analysis of full-length TRABID. Empty HA vector and HA-tagged full-length TRABID were immunoprecipitated from HEK293 cells and the precipitates were incubated with tetraUb chains for 1 hr. Western blotting analysis with  $\alpha$ -Ub and  $\alpha$ -HA shows TRABID-mediated cleavage of K63 chains only.

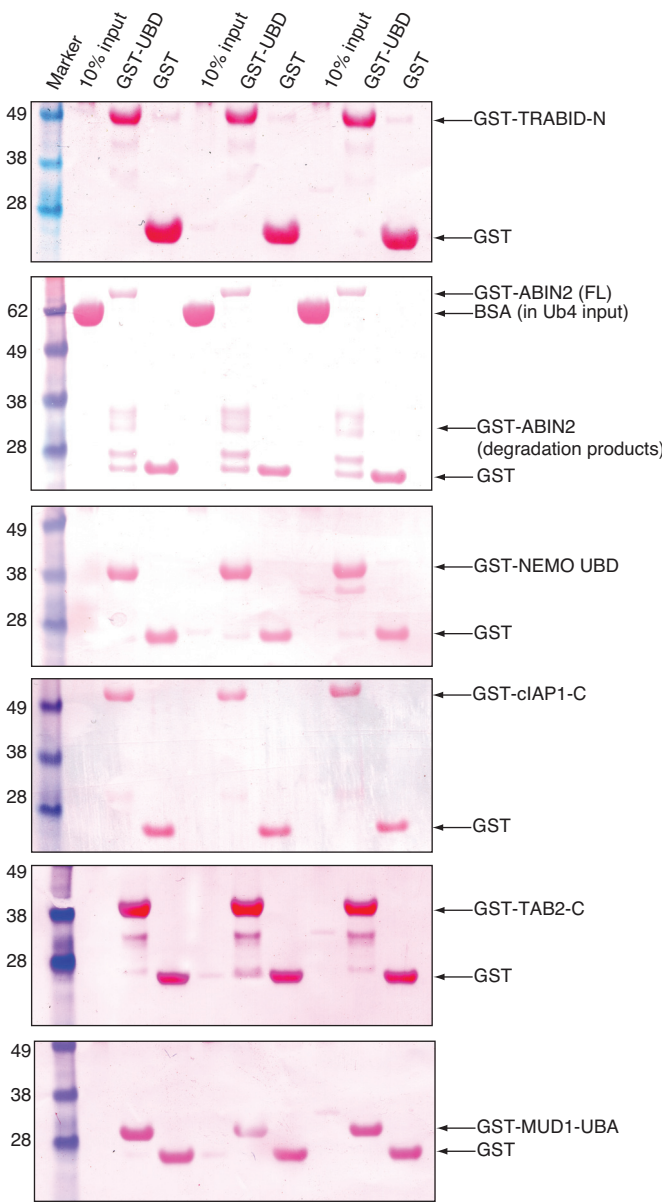


Supplementary Figure 1

**A****B**

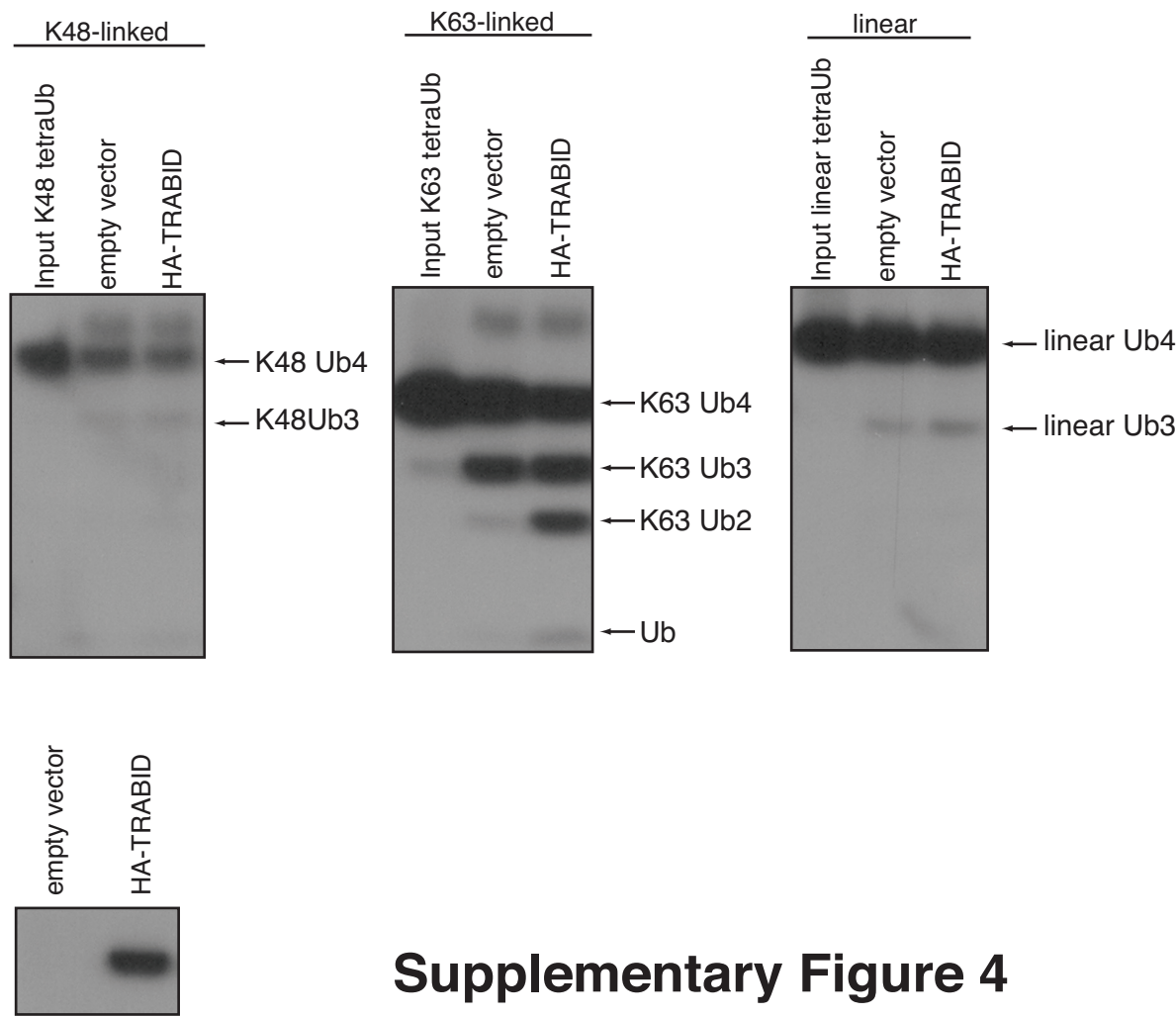
**Supplementary Figure 2**

K48-linked      K63-linked      linear



**Supplementary Figure 3**

# A FL - TRABID



**Supplementary Figure 4**